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Different Proteomics of Ca^{2+} on SA-induced Resistance to *Botrytis cinerea* in Tomato

LI Linlin [a,b], GUO Peng [a], JIN Hua [a], and LI Tianlai [b,*]

[a] *College of Environment and Resource, Dalian Nationaltities University, Dalian, Liaoning 116605, China*

[b] *College of Horticuture, Shenyang Agriculture University, Shenyang, Liaoning 110866, China*

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Abstract

This study aims to comprehensively study the effects of Ca^{2+} on the SA-induced resistance *Botrytis cinerea* in tomato through proteomics analysis. A proteomic approach was used to uncover the inducible proteins of tomato in the susceptible tomato cultivars 'L402' against *Botrytis cinerea* after salicylic acid (SA) and a combination treatment of $CaCl_2$ and SA. The results showed that the use of combination treatment of $CaCl_2$ and SA significantly enhanced tomato resistance against *Botrytis cinerea*. In total, 46 differentially expressed protein spots from 2-DE gel maps were detected, of which 41 were identified by mass spectrometry. All the identified proteins were categorized into eight groups according to their putative functions: defense response (14.00%), antioxidative protein (9.75%), photosynthesis (24.39%), molecular chaperone (4.88%), energy (17.01%), metabolism (21.95%), protein synthesis (4.88%) and signal transduction (0.2%). Of the proteins in the eight function groups, the effect of stress/defense and reactive oxygen species on Ca^{2+}-regulated SA-induced resistance may be the most important one in induced resistance by RT-PCR. The expression level of pathogenesis-related proteins (PRs) and chitinase was upregulated by a combination treatment of $CaCl_2$ and SA. The characterization of these proteins greatly helped to reveal the induced proteins involved in the regulation of Ca^{2+} on SA-induced resistance to *Botrytis cinerea*. In the combination treatment of $CaCl_2$ and SA, the defense response and antioxidative protein were clearly upregulated much more than SA alone or the control treatment by the method of proteomics and RT-PCR. The present findings suggest that susceptible tomato cultivars treated by the combination treatment of $CaCl_2$ and SA might possess a more sensitive SA signaling system or effective pathway than SA treatment alone. In addition, results indicated that SA could coordinate other cellular activities linked with photosynthesis and metabolism to facilitate defense response and recovery, indicating that the self-defense capability of tomato was improved by the combination treatment of $CaCl_2$ and SA.

Keywords: tomato; calcium; salicylic acid; *Botrytis cinerea*; proteomic; induced resistance

1. Introduction

Tomato (*Solanum lycopersicum* L.) is an important vegetable crop, and worldwide is the second most important vegetable crop next to potato. Present world production is about 100 million tons of fresh fruit from 3.7 million ha (Food and Agriculture Organization of the United Nations, 2001). However, its yield and quality are seriously compromised by infectious diseases caused by various fungal, bacterial, and viral diseases. Gray mold (https://en.wikipedia.org/wiki/Botrytis_cinerea), caused by the necrotrophic fungus *Botrytis cinerea*, is one of the most serious diseases of tomato. In China, gray mold is responsible for losses of over 30% of tomato yield in processing facilities (Yao et al., 2011). Control of the disease mainly depends on breeding resistant cultivars and using chemical fungicides. Breeding for resistance is the most economical method by which to control infection (Basnet et al., 2013). However, the frequent occurrence of new races of *Botrytis cinerea* has reduced the effectiveness of resistant cultivars in the greenhouse. Meanwhile, environmental concerns call for strict regulations on the use of chemical fungicides (Wahab, 2015). Development of new disease control strategies based on innate plant defense mechanisms may offer a promise for less crop loss due to *Botrytis cinerea*.

Plants have evolved a wide variety of inducible defense mechanisms that can be activated by a variety of biotic and abiotic stimuli aside from their basal physical and chemical barriers (Bari and

* Corresponding author. Tel.: +86 24 88487004.

E-mail address: tianlaili@126.com

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Jones, 2009; Justyna and Ewa, 2013). Salicylic acid (SA) is one of the signaling molecules associated with hypersensitive response (HR) and systemic acquired resistance (SAR); it can effectively induce the enhancement of enzymatic activity related to defensive processes (e.g. β-1, 3-glucanase and chitinase activities) (Vlot et al., 2009), increase total phenolic content, and cause immediate production of reactive oxygen species (ROS) (Yun and Chen, 2011), all three of the prior items to enhancement of plant resistance against a wide range of pathogens (Torres, 2009; Liu et al., 2012). In particular, the genetic role of SA in the activation of defense responses against biotrophic and hemibiotrophic pathogens has been widely recognized (Guru et al., 2007; Loake and Grant, 2007; Pieterse et al., 2009; Yuan et al., 2013), but the reports on the genetic role of SA in necrotrophic pathogens have been limited. Calcium is one of the important 'second messages'; some studies have shown that induced resistance of plants under biotic stress can be alleviated by exogenous Ca^{2+} (Lecourieux et al., 2006; Shao et al., 2008; Serrano et al., 2012; Downie, 2014). Treatment with Ca^{2+} can enhance the peroxidase activities, suggesting a physical cell wall strengthening and/or the generation of antimicrobial compounds that limit fungal development (Anna et al., 2005; Clark, 2013; Xu et al., 2013).

The role of Ca^{2+} in regulating SA in induction of tomato innate resistance against *Botrytis cinerea* infection has been widely documented in our lab (Li et al., 2012, 2015). However, there is little published information available on the mechanism of Ca^{2+} on SA in induction of disease resistance in the level of proteomic (Torres, 2009; Vlot et al., 2009; Liu et al., 2012; Yuan et al., 2013). In this study, we were interested in the identification and functional analysis of differently expressed proteins by various treatments. Thus, we investigated and analyzed the difference of the expressed proteins in the control, SA treatments, and a combination treatment of $CaCl_2$ and SA using 2-DE followed by MALDI™ TOF/TOF. The aim was to further explain the molecular mechanism of the biological process at the proteomic level. The present study offers new insights into the physiological mechanism involved in the regulation of Ca^{2+} on SA-induced resistance to *B. cinerea* and provides theoretical evidence for better disease control of tomato and other vegetables.

2. Materials and methods

2.1. Plant materials and treatment

The seeds of the tomato cultivar *S. lycopersicum* L. 'L402', a popular variety in Northeast China, are susceptible to *B. cinerea*. *S. lycopersicum* L. 'L402' seeds were germinated and grown in 12 cm^2 nutrition pots in heated greenhouses (average day/night temperatures, 25 °C /15 °C) with natural light and a relative humidity of 60% during April 2014 at the Shenyang Agricultural University. The plants were watered according to normal cultivation management.

The tomato plants were divided into three groups at the five-leaf stage, wherein each group contained 30 pots (3 biological replications with 10 plants per replication). Tomato 'L402' plants were given three treatments: the control, the SA treatment, and the combination treatment of $CaCl_2$ and SA (Ca + SA). The control treatment was foliar-sprayed ddH_2O; the SA treatment was foliar-sprayed exogenous 2 mmol · L^{-1} SA; and the Ca + SA treatment was foliar-sprayed ddH_2O and 8 mmol · L^{-1} $CaCl_2$ immediately followed by application of exogenous 2 mmol · L^{-1} SA. After three days of treatments, all the seedlings were inoculated with *B. cinerea* spores by placing 5 mL of a suspension of 10^6 spores · mL^{-1} in 2% glucose solution. Plants tissue samples were frozen using liquid nitrogen at 48 h days postinfection (DPI) and stored at −86 °C before being used to extract protein and mRNA. The disease survey was performed according to the methods of Fang (1998).

2.2. Extraction of secreted protein and mRNA from treated leaves

Proteins were extracted from leaves according to the method described by Lu et al. (2013). Three replicates of the treated seedlings were placed in liquid nitrogen, transferred to a pre-chilled mortar and under liquid nitrogen, and were ground into a fine powder using a pestle. Subsamples, weighing 3 g, were extracted using 30 volumes of pre-chilled 10% TCA–0.07% DTT/acetone buffer and precipitated overnight at −20 °C. Centrifugation was performed at 13 000 g for 60 min at 4 °C, then the supernatant was removed, and 30 volumes of pre-chilled 10% TCA–0.07% DTT/acetone buffer were added. The mixture was allowed to precipitate for 60 min, and then centrifuged at 13 000 g for 60 min at 4 °C. This step was repeated a minimum of four times. At last, the precipitant was simply air-dried at 4 °C for 5 min.

2.3. RNA isolation and validation of expression profiles reverse transcription

Total RNAs were extracted using TRIzol® reagent (Thermo Fischer Scientific, Waltham, MA, USA). The reverse transcription (RT) primers were designed following Chen et al. (2005) and Varkonyi-Gasic et al. (2007). The RT reactions were performed using M-MLV Reverse Transcriptase (Takara Bio Inc., Tokyo, Japan) as directed by the manufacturer. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the QuaniFast SYBR Green PCR Master Mix kit (Qiagen, Valencia, CA, USA) for qRT-PCR analysis using Applied Biosystems™ ABI PRISM® 7500 Sequence Detection System and its associated software (Thermo Fischer Scientific, Waltham, MA, USA). Primers were designed from the peptide sequences obtained after mass analysis according to NCBI and tomato databases (https://solgenomics.net/). Amplification was initiated with a denaturation step of 10 s at 95 °C, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. All reactions were performed in triplicate, and negative controls (no template and no RT) were included for each gene. The relative mRNA levels for each miRNA gene from various treatment samples were quantified with respect to the internal *Q96483* RNA standard. At least two independent RNA isolations were used for cDNA synthesis, with two biological replicates and three technical replicates for qRT-PCR analysis of each cDNA sample.

2.4. 2-DE analysis

All proteins were redissolved in rehydration buffer [9 mol · L^{-1} urea, 2 mol · L^{-1} thiourea, 4% CHAPS, 1% dithiothreitol (DTT), 1% PMSF] for 2-DE analysis. The protein content in the extracts was measured by the Bradford method using bovine serum albumin as the standard. The volume containing 1 mg total soluble protein

was mixed with the rehydration buffer containing 2% (v/v) pH 4–7 IPG buffer to a final volume of 450 mL. Electrophoresis consisted of eight steps: (1) 50 V for 15 h; (2) 100 V for 3 h, rapid; (3) 250 V for 3 h; (4) 500 V for 3 h; (5) 1 000 V for 2 h; (6) 10 000 V for 2 h; (7) 10 000 V for 160 000 Vhs; (8) 500 V for 24 h.

The entire procedure was performed at 20 °C. The IPG strips were equilibrated for SDS-PAGE in 10 mL of equilibration buffer [2% w/v SDS, 6 mol · L^{-1} urea, 20% v/v glycerol, 0.05 mol · L^{-1} Tris-HCl (pH 8.8)] containing 2% w/v DTT for 15 min followed by a second equilibration step of 15 min in the same equilibration buffer containing 2.5% w/v iodoacetamide. The equilibrated strips were loaded on 12.5% SDS-polyacrylamide gels. The electrophoresis conditions were 10 mA · gel^{-1} for 30 min and 50 mA · gel^{-1} for 5 h at 15 °C. The gels were stained with silver. The separated proteins were visualized by silver diamine-staining as described by Yan et al. (2000). Protein patterns in the gels were recorded as digitized images using a UMAX Powerlook 2100XL scanner (resolution 300 DPI): (1) spot detection, (2) gel matching, and (3) spot quantitation. This normalization method divided each spot abundance value by the sum of total spot abundance values to obtain the individual relative spot abundance. Relative intensity abundance of mature and germinated seeds (three replicate samples for each group) was compared using *t*-test. A two-fold or higher ($P \leq 0.05$) change in spot abundance value was set as the threshold, indicating a significant change. 2-DE was performed according to the method of Elise et al. (2006).

2.5. Tryptic digestion

Protein spots were cut from the gels then destained for 20 min in 30 mmol · L^{-1} potassium ferricyanide/100 mM sodium thiosulfate (1:1, v/v). They were then washed in Milli-Q water until the gels were destained. The spots were kept in 0.2 M NH_4HCO_3 for 20 min and followed by lyophilization. Each spot was digested overnight in 2 μL of 12.5 ng · μL^{-1} trypsin in 0.1 M NH_4HCO_3. The peptides were extracted three times with 50% ACN, 0.1% TFA.

2.6. Protein identification by mass spectroscopy

Spots of interest were manually cut out and placed in 1.5 mL centrifuge tubes. Mass spectroscopy (MS) was performed with a 4 800 MALDI TOF/TOF™ Analyzer (Thermo Fischer Scientific, Waltham, MA, USA). Monoisotopic peak masses were automatically determined within the mass range of 800–4 000 Da with a minimum signal-to-noise (S/N) ratio of 10 and a local noise window width of $m/z = 250$. Eight of the most intense ions with an S/N [50] were selected as precursors for MS/MS acquisition, excluding common trypsin autolysis peaks and matrix ion signals. MS together with the MS/MS spectra was searched using Applied Biosystems™ GPS Explorer™ and MASCOT® software as the database search engine with particular parameter settings. Only proteins with CI of 95% or greater were considered to be positively identified.

2.7. Statistical analysis

Data were analyzed using OriginPro v.7 (OriginLab Corporation, Northampton, MA, USA) and DPS v.3.01 statistical packages (Refine Information Tech, Hangzhou, Zhejiang University, China). Measurements were performed on randomly selected samples from all three replicates of each treatment. Duncan's multiple range tests were used to test for significant differences between treatment groups at $P \leq 0.05$.

3. Results

3.1. Establishment of pathogen infection in leaves

The fungi-inoculated plants were scored for the disease index and resistance category following the standard of Fang (1998). The disease index was calculated after 5 d of being infected with *B. cinerea*. The SA-only and the combination treatment of Ca + SA disease index were 46.95 and 38.53 respectively; which was significant ($P \leq 0.05$) and lower than the control (74.85). Furthermore, Ca + SA reduced the disease index on the base of SA.

3.2. Comparative analysis of protein maps

The general protein patterns from 2-DE gels were built for each treatment in the pH range of 4–7. The 2-DE gels revealed approximately 621 protein spots in the control treatment (Fig. 1, A), 675 protein spots in the SA treatment (Fig. 1, B) and 697 protein spots in the combination treatment Ca + SA (Fig. 1, C). After analysis with PDQuest 7.3.1 software, MALDI™ TOF/TOF was used to analyze a total of 46 excised protein spots whose abundance changed by two-fold or higher. All 41 successfully identified protein spots are shown in Fig. 2. The abundant changes of protein spots reflected on changes of the amount. There were 17 protein spots that were upregulated (1, 3, 5, 7, 1621, 1805, 2011, 2012, 3211, 3448, 4011, 4308, 5210, 5504, 5810, 6107 and 7711) in the SA-only treatment and Ca + SA treatment, the expression level of the protein spots was higher in Ca + SA treatment than the SA-only treatments. Also, only three protein spots (2, 2123 and 23251) were upregulated in the Ca + SA treatment. The six protein spots (108, 1807, 2005, 2006, 6403 and 23207) were downregulated in the SA-only treatment and in the combination treatment Ca + SA. Moreover, there were nine new spots that appeared (12, 1101, 1307, 1402, 2010, 2207, 6001, 23325, 23447) in the SA and Ca + SA treatments, and the protein expression level was clearly higher in Ca + SA.

3.3. Proteomic identification and functional categorization

Using the NCBI search, a total of 41 spots were identified with an identification success rate of 77.4%. Several proteins remained unidentified because the number and/or intensity of the fragment ions obtained by MS/MS were insufficient for a significant hit. Table 1 shows the list of the proteins that could be identified at a significant level of confidence by MS/MS; the average fold change represents the ratio of change of spot intensity in comparison with the control, the spots showing that fold changes which were closed to the significant level are also highlighted.

A few proteins appeared as multiple spots with different pI and Mr as was the case for Rubisco and which had been previously observed in other species (Meunier et al., 2005; Kuczyńska et al., 2012). Proteins were classified into 8 functional categories, including defense response ($n = 6$), antioxidative system protein ($n = 4$), photosynthesis ($n = 10$), molecular chaperone ($n = 2$), energy ($n = 7$), metabolism ($n = 9$), protein synthesis ($n = 2$), signal transduction ($n = 1$) (Table 1).

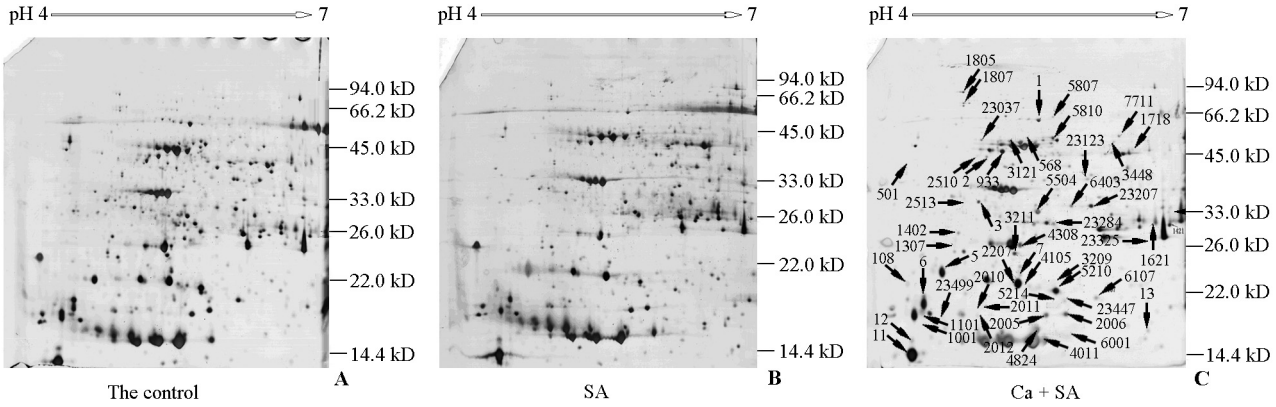

Fig. 1 Representative 2-DE patterns of proteins from tomato leaves (5th leaves of seedlings) treated with Milli-Q water (as the control, A), SA only (B), and the combination treatment of Ca + SA (C)

Black arrows indicate the positions of differentially expressed protein spots compared with their respective controls.

Fig. 2 2-DE gels showing differentially expressed protein spots in two treatments of the control, SA only, and the combination treatment Ca + SA 48 h after inoculation with *B. cinerea*

The red boxes indicate proteins whose expression changed in response to any of the treatments. The relative locations of these protein spots are indicated in Fig. 1.

Table 1 Identification of differentially expressed proteins from tomato leaves infected with *B. cinerea* after treatment with control, SA, or Ca + SA

Function	Spot no.	Accession no.	Protein name	Species	Score	Mr/kD	pI	Peptides matched	Sequence coverage/%	Protein score C.I. /%
Defense	6	gi|17017306	SGT1	*Hordeum vulgare*	60	41.14	4.93	10	68	99.41
response protein	4105	gi|544007	Acidic 26 kD endochitinase	*Solanum lycopersicum*	583	28.01	5.93	6	53	100.00
	5504	gi|544007	Acidic 26 kD endochitinase	*Solanum lycopersicum*	244	28.01	5.93	9	27	100.00
	5214	gi|58531054	Putative pathogenesis-related protein	*Capsicum chinense*	103	17.27	5.22	14	58	100.00
	6107	gi|131026	Pathogenesis-related protein STH-2	*Solanum lycopersicum*	85	17.40	5.66	10	59	99.74
	23325	gi|157830095	Pathogenesis-related protein 5d	*Nicotiana tabacum*	286	23.66	7.34	5	30	100.00
Antioxidant system	2005	gi|313103751	Chain A, Zinc-reconstituted tomato chloroplast superoxide dismutase	*Solanum lycopersicum*	683	15.73	5.30	5	57	100.00
	2006	gi|3334337	Superoxide dismutase [Cu-Zn] 2	*Nicotiana tabacum*	382	15.35	5.65	5	55	100.00
	6403	gi|73543248	Cytosolic ascorbate peroxidase 1	*Solanum lycopersicum*	256	27.73	5.61	7	34	100.00
	23207	gi|73543248	Cytosolic ascorbate peroxidase 1	*Solanum lycopersicum*	114	27.74	5.61	9	51	100.00
Photosynthesis-related enzymes	3	gi|132104	Ribulose-1,5-bisphosphate carboxylase, small subunit	*Solanum lycopersicum*	248	20.44	6.73	6	39	97.34
	501	gi|255545796	Cytochrome C oxidase polypeptide vib, putative	*Ricinus communis*	82	19.23	4.28	4	37	99.42
	1101	gi|170502	Ribulose-1,5-bisphosphate carboxylase, small subunit	*Solanum lycopersicum*	268	20.56	7.57	6	30	100.00
	2011	gi|170504	Ribulose-1,5-bisphosphate carboxylase, small subunit precursor	*Solanum lycopersicum*	445	20.52	7.57	7	37	100.00
	2012	gi|132104	Ribulose-1,5-bisphosphate carboxylase, small subunit precursor	*Solanum lycopersicum*	248	20.45	6.73	7	38	100.00
	4011	gi|170504	Ribulose-1,5-bisphosphate carboxylase, small subunit precursor	*Solanum lycopersicum*	510	20.48	6.59	8	54	100.00
	4308	gi|170388	Chlorophyll a/b-binding protein	*Solanum lycopersicum*	117	26.68	5.82	5	32	100.00
	4824	gi|170504	Ribulose-1,5-bisphosphate carboxylase, small subunit precursor	*Solanum lycopersicum*	391	20.49	6.59	7	44	100.00
	6001	gi|132104	Ribulose-1,5-bisphosphate carboxylase, small subunit precursor	*Solanum lycopersicum*	424	20.45	6.73	8	47	100.00
	23499	gi|170502	Ribulose-1,5-bisphosphate carboxylase, small subunit precursor	*Solanum lycopersicum*	191	20.56	7.57	6	58	100.00
Molecular	1805	gi|2654208	Heat shock protein 70	*Spinacia oleracea*	676	76.27	5.19	16	48	100.00
chaperones	5810	gi|68566314	Elongation factor TuB, chloroplastic	*Solanum lycopersicum*	583	52.77	5.95	13	50	100.00
Energy	1	gi|159227262	ATP synthase beta subunit	*Solanum humboldti*	123	35.90	5.54	12	66	100.00
	12	gi|73543248	ATP synthase beta submit	*Micromonas pusilla*	256	18.36	5.44	9	52	99.93
	108	gi|302769494	Phosphoglycerate kinase	*Arabidopsis thaliana*	53	70.31	6.92	7	53	100.00
	1402	gi|416681	ATP synthase delta chain, chloroplastic	*Solanum lycopersicum*	123	26.77	8.96	5	27	100.00
	2207	gi|146454658	Cytochrome B6-F complex iron sulfur subunit 2	*Sonneratia ovata*	128	16.83	6.04	14	30	100.00
	3211	gi|48209968	ATP synthase D chain, mitochondrial, putative	*Solanum demissum*	186	19.80	5.34	10	37	100.00
	23251	gi|48209968	ATP synthase D chain, mitochondrial	*Solanum demissum*	186	19.80	5.34	10	35	100.00
Metabolism	2	gi|238563983	Chloroplast sedoheptulose-1,7-bisphosphatase	*Solanum lycopersicum*	370	43.01	6.07	15	42	100.00
	7	gi|238563983	Chloroplast sedoheptulose-1,7-bisphosphatase	*Solanum lycopersicum*	154	43.02	6.07	4	27	100.00
	1307	gi|37625527	Miraculin-like protein	*Solanum palustre*	137	23.71	5.50	9	43	100.00
	1421	gi|56562177	Carbonic anhydrase	*Solanum lycopersicum*	240	34.85	6.67	16	47	100.00
	1807	gi|82400156	Phosphoglycerate kinase precursor-like protein	*Solanum tuberosum*	202	50.63	7.68	9	52	100.00
	3448	gi|327198779	Glyceraldehyde-3-phosphate dehydrogenase	*Solanum tuberosum*	145	48.54	7.06	10	84	100.00
	5210	gi|297809307	Flavonol synthase	*Nicotiana tabacum*	286	32.65	5.00	4	26	93.00
	7711	gi|327198779	Glyceraldehyde-3-phosphate dehydrogenase	*Solanum tuberosum*	426	48.54	7.06	15	40	100.00
	23123	gi|61969082	Putative ferredoxin-NADP reductase	*Solanum peruvianum*	589	35.47	7.71	13	55	100.00
Protein synthesis	5	gi|93280152	50S ribosomal protein L12	*Solanum lycopersicum*	277	17.67	5.99	5	30	100.00
	1621	gi|307103458	ATP-binding cassette superfamily	*Chlorella variabilis*	74	41.21	9.58	14	48	96.07
Signal transduction	23447	gi|14010483	Pto-like kinase SG5	*Phaseolus vulgaris*	74	19.22	9.19	7	33	96.25

3.4. Possible roles of the proteins in Ca^{2+}-regulated SA-induced resistance to B. cinerea

Putative protein functional classification was assigned on the basis of similarity, with the aim of identifying the biological processes associated with proteins identified by 2-DE proteomics. All the identified proteins were categorized into eight groups according to their putative functions: defense response (14.00%), antioxidative protein (9.75%), photosynthesis (24.39%), molecular chaperone (4.88%), energy (17.01%), metabolism (21.95%), protein synthesis (4.88%) and signal transduction (0.2%). Based on their putative functions in other plants, possible roles were assigned to the proteins that showed upregulated to downregulated expression during Ca^{2+}-regulated SA-induced resistance to *B. cinerea* infection. We considered how these proteins might be involved in the induced process.

3.4.1. Defense response protein

The CHI and PR were newly appearing proteins only in the Ca + SA treatment (No. 4105 and No. 5214) as also shown in Fig. 2. No. 4105, 5504 (also named CHI) and No. 6107 (STH-2) existed in three treatments, and the order of expression level decreased as the Ca + SA > SA > control. No. 23325 was not expressed in the control; it belonged to the newly appearing protein in SA and Ca + SA treatments, and the expression level was stronger in SA-only than the Ca + SA treatment.

3.4.2. Antioxidant systems

Plants have a set of enzymes and redox metabolites that carry out ROS detoxification and are known as antioxidant systems (Bowler and Fluhr, 2000; Ganesan and Thomas, 2001). In our study, four proteins were identified: zinc-reconstituted tomato chloroplast superoxide dismutase (SOD, No. 2005), superoxide dismutase [Cu-Zn] 2 (Cu-Zn SOD, No. 2006), cytosolic ascorbate peroxidase 1 (CAT, No. 6403 and No. 23207). SOD, Cu-Zn SOD, and CAT were lower in SA and Ca + SA treatments compared with the control. Meanwhile, the protein expression level was weaker in Ca + SA than in the SA-only treatment. CAT (No. 6403) disappeared only in Ca + SA treatment. In the SA-only treatment, the CAT expression level was weaker than in that of the control.

3.4.3. Photosynthesis-related enzymes

Ribulose-1,5-bisphosphate carboxylase (No. 3, 2012 and 6001), ribulosebisphosphate carboxylase (No. 1101, 4011) and chlorophyll a/b-binding protein (No. 4308) expressed stronger in the SA-only and Ca + SA treatment than in the control. In the SA-only treatment, the protein expression level was weaker than the combination treatment Ca + SA. Protein spot No. 2011 was not expressed in the control, and the protein expression level was stronger in Ca + SA than the SA-only treatment. No. 501 and 23499 were also newly appearing protein which appeared only in the Ca + SA treatment.

3.4.4. Molecular chaperones

Protein spots No. 1805 and 5810 were the heat shock protein 70 and elongation factor TuB, respectively. Heat shock proteins (HSPs) are generally classified according to their approximate molecular size, and are structurally and functionally diverse; some are constitutively expressed, while others are stress-induced (Mayer and Bukau, 2005; Brodsky and Gabriela, 2006; Carmen et al., 2006; Elise et al., 2006; Powers and Paul, 2007). Elongation is the most rapid step in translation in eukaryotes, and the rate is about two amino acids per second. Elongation factors play a role in orchestrating the events of this process and in ensuring the 99.99% accuracy of translation at this speed. In our study, heat shock protein 70 and elongation factor TuB expressed stronger in the SA-only and Ca + SA treatment than in the control, and the protein expression level was weaker in the SA-only treatment than in Ca + SA treatment.

3.4.5. Energy

Protein spot No. 1 was the ATP synthase beta subunit, No. 1402 was the ATP synthase delta chain, No. 2207 was the cytochrome B6-F complex iron sulfur; No. 3211 and 23251 were ATP synthase D chain. Nos. 1 and 1402 were the newly appearing proteins only in the SA and Ca + SA treatments; the protein expression levels were stronger in the Ca + SA treatment than in the SA-only treatment. Protein spots 2207, 3211, and 23251 expressed stronger in the SA-only and Ca + SA treatments than in the control, and the expression level was stronger in Ca +SA treatment than in the SA-only treatment. These protein expression levels were significantly upregulated both in the SA-only and Ca + SA treatment at 48 h after *B. cinerea* inoculation.

3.4.6. Metabolism

Spots 2 and 7 were sedoheptulose-1,7-bisphosphatase (SBPase). It has also been suggested that SBPase plays a vital role in regulating the Calvin cycle pathway. Spot 1421 was carbonic anhydrase; 18 and 1807 were phosphoglycerate kinase precursor-like proteins; 3448 and 7711 were glyceraldehyde-3-phosphate dehydrogenases; 5210 was flavonol synthase; 23123 was putative ferredoxin-NADP reductase. The upregulation included 2, 7, 1421, 3448, 7711, and 23123; and downregulation was 1807. No. 23123 expression was stronger in Ca + SA treatment than in the control and SA-only treatments.

3.4.7. Protein biosynthesis

Ca^{2+} and SA positively regulated the expression of two proteins associated with protein synthesis, including ribosomal protein (No. 5) and ATP-binding cassette superfamily (No.1621).

3.4.8. Signal transduction

Protein No. 23447 was identified as Pto-like kinase SG5, which notably increased in the SA-only and in the Ca + SA. The expression level was significantly stronger in Ca + SA treatment than the SA-only treatment, but no significant expression was seen in the control.

3.5. Transcript accumulation patterns for 12 candidate proteins

RT-PCR was used to analyze the changes in gene expression at the mRNA level of 12 identified proteins involved in energy metabolism, antioxidant enzyme system, protein kinase system, and defense against stress (Fig. 3). The mRNA levels of nine transcripts increased under the SA-only and Ca + SA treatments compared with the control, and the expression level of the Ca + SA treatment was more strengthened than the SA-only treatment.

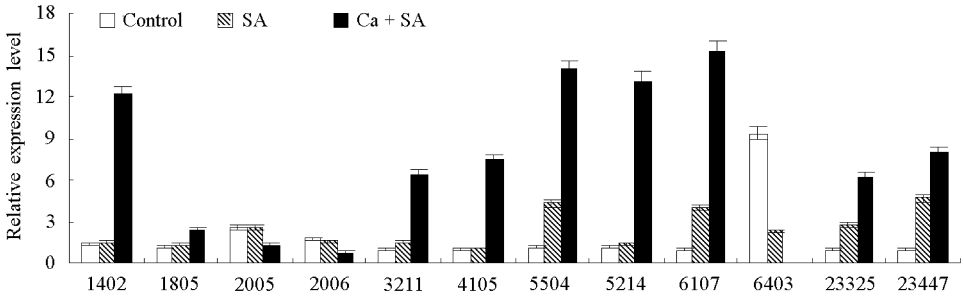

Fig. 3 RT-PCR analysis of transcript levels of differentially expressed proteins with three treatments
A single concentration of cDNA was also used for amplification with ACTIN (Q96483, actin) primers.

Antioxidant enzyme system gene expression was decreased in the SA-only and the Ca + SA compared with the control.

4. Discussion

Proteome is complicated and varied, which is closely associated with protein performing its function (Hao et al., 2011). In cell, except housekeeping protein, most of the proteins expression level will vary as the changes of cell function performance and/or environment (Shao et al., 2008). A proteomic approach was used to identify host proteins altering in abundance during *B. cinerea* infection of a susceptible tomato cultivar 'L402' after SA and a combination treatment of $CaCl_2$ and SA. Of all the identified proteins, there were 10 proteins that refer to antioxidant enzyme system proteins and defense-related proteins, 10 proteins were photosynthesis-related proteins, 16 proteins were energy and metabolite-related protein, 5 proteins were of other-function protein.

Nos. 2005, 2006, 6403, 23207 were identified as antioxidant enzyme system proteins, and they were simultaneously downregulated by SA and Ca + SA, suggesting that Ca^{2+} might regulate SA-induced and boost ROS level in tomato seedlings to defend *B. cinerea* infections. Consistent with this notion, Ca + SA and SA-only treatments led to significant accumulation of O_2^- and H_2O_2 in reasonable level in our previous studies (Li et al., 2012, 2015). Similar results have been reported in various plants after treatment with SA only (Rao et al., 1997; Ganesan and Thomas, 2001; Torres, 2009). The synthesis and accumulation of pathogenesis-related proteins are ubiquitous plant responses to an exogenous elicitor or pathogenic infection (Liao et al., 2009; Alexandersson et al., 2013; Heyno et al., 2013; Belkadhi et al., 2014; Noam et al., 2014). However, SA plays a crucial role in plant defense, and exogenous applications result in the induction of *pathogenesis related* (*PR*) genes and enhanced resistance to a broad range of pathogens (Grant and Lamb, 2006). Various proteins such as pathogenesis-related protein 5, SGT1 and putative intracellular pathogenesis-related protein, as identified in this study, are known to be upregulated in the SA-only and Ca + SA treatment in response to fungal elicitation *B. cinerea*, which is in agreement with previous reports on exogenous SA treatment (Liao et al., 2009; Vlot et al., 2009; Kundu et al., 2011). However, Ca + SA treatment enhanced the protein expression level on the basis of SA treatment. The results from this research suggest that Ca^{2+} might regulate SA-induced chitinase and pathogenesis-related protein biosynthesis to attenuate *B. cinerea* infections. The above findings indicate that the Ca^{2+} enhanced the protective effect of SA on tomato seedlings against *B. cinerea* infection by coordinating oxidative burst, decreasing the cellular level of ROS-scavenging antioxidant enzymes, and activating the defense response, including chitinase activity improvement, pathogenesis-related protein biosynthesis, and cell wall strengthening.

The upregulation (Nos. 3, 2012, 4011, 4308) and newly appearing proteins (Nos. 1101, 2011, 4824, 6001 and 23499) were photosynthesis related proteins and suggest an enhancement of photosynthetic rate following infection. Presumably, it was advantageous for the plant to maintain photosynthesis to produce assimilates for defense reaction. The induction of defense has been shown to be cost-intensive (Salvucci, 2008); the necrotrophic pathogen kills the host tissue and feeds on the dead tissue (Berkowitz, 2013). Therefore, it could be said that changes in assimilate levels might influence the defense response of the host plant against the pathogen. Previous studies have shown that a range of stress conditions (e.g. heat stress, cold, UV light) could cause decreased photosynthesis, including pathogen infection (Mahmood et al., 2006; Amey et al., 2008). It is interesting to note that the SA-only treatment and the Ca + SA significantly increased the cellular content of the nine proteins related to photosynthesis, including ribulose-1,5-bisphosphate carboxylase, ribulosebisphosphate carboxylase, and chlorophyll a/b-binding protein. Our proteomic data were agreeable with the previous studies that showed SA could alleviate decrease in plant photosynthesis under various stress conditions (Wang et al., 2010; Nazar et al., 2011). It was not surprising that enhanced ATP production was also coordinated to keep up with the activated photosynthesis machinery and the Calvin cycle to produce assimilates for the defense reaction (Nishizawa and Hirai, 1989; Yan et al., 2010). ATP synthase proteins (Nos. 1, 1402, 3211, 23251) were found to improve photosynthetic capacity (Feng et al., 2014) and adapt and regulate tolerance resistance to tomato *B. cinerea* in this study. It has been reported that ribosomal protein and ATP-binding cassette superfamily are associated with protein synthesis and played an important role in plant immunity (Nishizawa and Hirai, 1989; Yan et al., 2010; Dangl et al., 2013). Keeping a reasonable level of photosynthesis would be important for plants during defense response as it is a costly process requiring not only energy but also various metabolites (Sun et al., 2014). And transcription factor and kinase SG5 played a

significant role in signal transduction pathways, such as ROS signaling and phytochrome signaling (Mucyn et al., 2009; Kalavacharla et al., 2011). It was reported that the Pto-like kinase that regulated plant immunity was repressed by its myristoylated N terminus (Ye et al., 2000; Andriotis and Rathjen, 2006). The kinase may play a subsidiary role in the process.

In the RT-PCR analysis, the mRNA levels of transcripts increased under the SA-only and Ca + SA treatments compared with the control, and the expression level of the Ca + SA treatment was more strengthened than the SA-only treatment except antioxidant enzyme system. Though the activities of the antioxidant enzyme system were increased under SA treatment, further more, these enzymes activities were increased in Ca + SA treatment in our previous results (Li et al., 2015). The *zinc-reconstituted superoxide dismutase*, *superoxide dismutase [Cu-Zn] 2*, and *cytosolic ascorbate peroxidase* genes showed an opposite tendency. Thus, the mRNA levels did not correspond with the protein levels. This was not surprising because the final amount and activity of a protein represents an accumulation regulatory event at their transcriptional, post-transcriptional, translational, and post-translational levels (Yan et al., 2001). Therefore, the validity of estimating gene expression levels using protein expression data requires further study.

The proteome profile of tomato and its characterization provided a foundation for understanding the molecular basis underlying in Ca^{2+} regulated SA-induced resistance to grey mold, and thus will open new avenues toward engineering tomato for improved resistance and enhanced yields value.

5. Conclusion

In the present study, SA-only and the combination treatment Ca + SA were demonstrated to be involved in the resistance of tomato plants. The combination treatment Ca + SA enhanced the expression of proteins involved in the defense response, photosynthesis, energy, molecular chaperone, metabolism, and signal transduction. The phenomenon suggests that exogenous Ca + SA treatment could induce *B. cinerea* resistance by improving enzyme activity in systems related to photosynthesis, energy, and stress defense in tomato plants. However, RT-PCR analyses showed some different results for the candidate proteins. In general, the exogenous combination treatment Ca + SA improves the *B. cinerea* resistance of plants via multiple systems that are regulated by multiple genes relating to various metabolic and signaling pathways. The research provided evidence of enhanced tomato resistance to *B. cinerea* mainly by the effect of exogenous combination treatment of $CaCl_2$ and SA on the pathogen related proteins, metabolic and antioxidative system activities of tomato. Further proteomic studies in this area are clearly warranted and are ongoing. The data imply that SA combined with Ca^{2+} may be an appropriate treatment choice for treatment of *B. cinerea*.

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